

METHODS FOR TREATING DISEASES CAUSED BY DEFICIENCIES OF  
RECOMBINANT  $\alpha$ -L-IDURONIDASE

This application claims priority to U.S. Patent Application Serial No. 09/711,205,  
5 filed on November 9, 2000, which is a continuation-in-part of U.S. Patent Application  
Serial No. 09/439,923, filed on November 12, 1999.

FIELD OF THE INVENTION

The present invention is in the field of molecular biology, enzymology,  
10 biochemistry and clinical medicine. In particular, the present invention provides a human  
recombinant  $\alpha$ -L-iduronidase, methods of large-scale production and purification of  
commercial grade human recombinant  $\alpha$ -L-iduronidase enzyme, and methods to treat  
certain genetic disorders including  $\alpha$ -L-iduronidase deficiency and  
mucopolysaccharidosis I (MPS I).

BACKGROUND OF THE INVENTION

Carbohydrates play a number of important roles in the functioning of living  
organisms. In addition to their metabolic roles, carbohydrates are structural components  
of the human body covalently attached to numerous other entities such as proteins and  
20 lipids (called glycoconjugates). For example, human connective tissues and cell  
membranes comprise proteins, carbohydrates and a proteoglycan matrix. The  
carbohydrate portion of this proteoglycan matrix provides important properties to the  
body's structure.

A genetic deficiency of the carbohydrate-cleaving, lysosomal enzyme  $\alpha$ -L-  
25 iduronidase causes a lysosomal storage disorder known as mucopolysaccharidosis I (MPS  
I) (Neufeld and Muenzer, pp. 1565-1587, in *The Metabolic Basis of Inherited Disease*,  
Eds., C.R. Scriver, A.L. Beaudet, W.S. Sly, and D. Valle, McGraw-Hill, New York  
(1989)) In a severe form, MPS I is commonly known as Hurler syndrome and is  
associated with multiple problems such as mental retardation, clouding of the cornea,  
30 coarsened facial features, cardiac disease, respiratory disease, liver and spleen  
enlargement, hernias, and joint stiffness. Patients suffering from Hurler syndrome usually  
die before age 10. In an intermediate form known as Hurler-Scheie syndrome, mental

function is generally not severely affected, but physical problems may lead to death by the teens or twenties. Scheie syndrome is the mildest form of MPS I. It is compatible with a normal life span, but joint stiffness, corneal clouding and heart valve disease cause significant problems.

5           The frequency of MPS I is estimated to be 1:100,000 according to a British Columbia survey of all newborns (Lowry, *et al.*, *Human Genetics* 85:389-390 (1990)) and 1:70,000 according to an Irish study (Nelson, *Human Genetics* 101:355-358 (1990)). There appears to be no ethnic predilection for this disease. It is likely that worldwide the disease is underdiagnosed either because the patient dies of a complication before the  
10       diagnosis is made or because the milder forms of the syndrome may be mistaken for arthritis or missed entirely. Effective newborn screening for MPS I would likely find some previously undetected patients.

          Except for a few patients which qualify for bone marrow transplantation, there are no significant therapies available for all MPS I patients. Hobbs, *et al.* (*Lancet* 2: 709-712  
15       (1981)) first reported that bone marrow transplantation successfully treated a Hurler patient. Since that time, clinical studies at several transplant centers have shown improvement in physical disease and slowing or stabilizing of developmental decline if performed early. (Whitley, *et al.*, *Am. J. Med. Genet.* 46: 209-218 (1993); Vellodi, *et al.*, *Arch. Dis. Child.* 76: 92-99 (1997); Peters, *et al.*, *Blood* 91: 2601-2608 (1998); Guffon, *et al.*, *J. Pediatrics* 133: 119-125 (1998)) However, the significant morbidity and mortality, and the need for matched donor marrow, limits the utility of bone marrow transplants. An  
20       alternative therapy available to all affected patients would provide an important breakthrough in treating and managing this disease.

          Enzyme replacement therapy has been considered a potential therapy for MPS I  
25       following the discovery that  $\alpha$ -L-iduronidase can correct the enzymatic defect in Hurler cells in culture, but the development of human therapy has been technically unfeasible until now. In the corrective process, the enzyme containing a mannose-6-phosphate residue is taken up into cells through receptor-mediated endocytosis and transported to the lysosomes where it clears the stored substrates, heparan sulfate and dermatan sulfate.  
30       Application of this therapy to humans has previously not been possible due to inadequate sources of  $\alpha$ -L-iduronidase in tissues.

For  $\alpha$ -L-iduronidase enzyme therapy in MPS I, a recombinant source of enzyme has been needed in order to obtain therapeutically sufficient supplies of the enzyme. The cDNA for the canine enzyme was cloned in 1991 (Stoltzfus, *et al.*, *J. Biol. Chem.* 267:6570-6575 (1992) and for the human enzyme in the same year. (Scott, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 88:9695-9699 (1991), Moskowitz, *et al.*, *FASEB J* 6:A77 (1992)). Following the cloning of cDNA for  $\alpha$ -L-iduronidase, the production of adequate quantities of recombinant  $\alpha$ -L-iduronidase allowed the study of enzyme replacement therapy in canine MPS I. (Kakkis, *et al.*, *Protein Expr. Purif.* 5: 225-232 (1994)) Enzyme replacement studies in the canine MPS I model demonstrated that intravenously-administered recombinant  $\alpha$ -L-iduronidase distributed widely and reduced lysosomal storage from many tissues. (Shull, *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 91: 12937-12941 (1994); Kakkis, *et al.*, *Biochem. Mol. Med.* 58: 156-167 (1996))

#### BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention features a method to mass produce human recombinant  $\alpha$ -L-iduronidase in large scale amounts with appropriate purity to enable large scale production for long term patient use of the enzyme therapy. In a broad embodiment, the method comprises the step of transfecting a cDNA encoding for all or part of an  $\alpha$ -L-iduronidase into a cell suitable for the expression thereof. In some embodiments, a cDNA encoding for a complete  $\alpha$ -L-iduronidase is used, preferably a human  $\alpha$ -L-iduronidase. However, in other embodiments, a cDNA encoding for a biologically active fragment or mutein thereof may be used. Specifically, one or more amino acid substitutions may be made while preserving or enhancing the biological activity of the enzyme. In other preferred embodiments, an expression vector is used to transfer the cDNA into a suitable cell or cell line for expression thereof. In one particularly preferred embodiment, the cDNA is transfected into a Chinese hamster ovary cell to create cell line 2.131. In yet other preferred embodiments, the production procedure features one or more of the following characteristics which have demonstrated particularly high production levels: (a) the pH of the cell growth culture may be lowered to about 6.5 to 7.0, preferably to about 6.8 - 7.0 during the production process, (b) as many as 2 to 3.5 culture volumes of the medium may be changed during each 24-hour period by continuous perfusion, (c) oxygen saturation may be optimized to about 40% but

may be as high as 80%, (d) macroporous cellulose microcarriers with about 5% serum in the medium initially, may be used to produce cell mass followed by a rapid washout shift to protein-free medium for production, (e) a protein-free or low protein-medium such as a JRH Biosciences PF-CHO product may be optimized to include supplemental amounts of one or more ingredients selected from the group consisting of: glutamate, aspartate, glycine, ribonucleosides, and deoxyribonucleosides; (f) a stirred tank suspension culture may be perfused in a continuous process to produce iduronidase.

In a second aspect, the present invention provides a transfected cell line, which features the ability to produce  $\alpha$ -L-iduronidase in amounts, which enable using the enzyme therapeutically. In preferred embodiments, the present invention features a recombinant Chinese hamster ovary cell line such as the 2.131 cell line that stably and reliably produces amounts of  $\alpha$ -L-iduronidase which enable using the enzyme therapeutically. In some preferred embodiments, the cell line may contain more than 1 copy of an expression construct. In even more preferred embodiments, the cell line expresses recombinant  $\alpha$ -L-iduronidase in amounts of at least 20 micrograms per  $10^7$  cells per day.

In a third aspect, the present invention provides novel vectors suitable to produce  $\alpha$ -L-iduronidase in amounts which enable using the enzyme therapeutically. In preferred embodiments, the present invention features an expression vector comprising a cytomegalovirus promoter/enhancer element, a 5' intron consisting of a murine C $\alpha$  intron, a cDNA encoding all or a fragment or mutein of an  $\alpha$ -L-iduronidase, and a 3' bovine growth hormone polyadenylation site. Also, preferably the cDNA encoding all or a fragment or mutein of an  $\alpha$ -L-iduronidase is about 2.2 kb in length. This expression vector may be transfected at, for example, a 50 to 1 ratio with any appropriate common selection vector such as pSV2NEO, to enhance multiple copy insertions. Alternatively, gene amplification may be used to induce multiple copy insertions.

In a fourth aspect, the present invention provides novel  $\alpha$ -L-iduronidase produced in accordance with the methods of the present invention and thereby present in amounts which enable using the enzyme therapeutically. The specific activity of the  $\alpha$ -L-iduronidase according to the present invention is in excess of 200,000 units per milligram protein. Preferably, it is in excess of about 240,000 units per milligram protein. The

molecular weight of the  $\alpha$ -L-iduronidase of the present invention is about 82,000 daltons, about 70,000 daltons being amino acid, and about 12,000 daltons being carbohydrates.

In a fifth aspect, the present invention features a novel method to purify  $\alpha$ -L-iduronidase. According to a first embodiment, a cell mass may be grown in about 5% serum-containing medium, followed by a switch to a modified protein-free production medium without any significant adaptation to produce a high specific activity starting material for purification. In one preferred embodiment, a three step column chromatography may be used to purify the enzyme. Such a three step column chromatography may include using a blue sepharose FF, a  $\text{Cu}^{++}$  chelating sepharose chromatography and a phenyl sepharose HP chromatography. In another preferred embodiment, an acid pH treatment step is used to inactivate potential viruses without harming the enzyme. Concanavalin A-Sepharose, Heparin-Sepharose and Sephacryl 200 columns are removed and Blue-Sepharose and copper chelating columns added to increase the capacity of the large scale purification process, to reduce undesirable leachables inappropriate for long term patient use, and to improve the purity of the product.

In a sixth aspect, the present invention features novel methods of treating diseases caused all or in part by a deficiency in  $\alpha$ -L-iduronidase. In one embodiment, this method features administering a recombinant  $\alpha$ -L-iduronidase or a biologically active fragment or mutein thereof alone or in combination with a pharmaceutically suitable carrier. In other embodiments, this method features transferring a nucleic acid encoding all or a part of an  $\alpha$ -L-iduronidase into one or more host cells *in vivo*. Preferred embodiments include optimizing the dosage to the needs of the organism to be treated, preferably mammals or humans, to effectively ameliorate the disease symptoms. In preferred embodiments, the disease is Mucopolysaccharidosis I (MPS I), Hurler syndrome, Hurler-Scheie syndrome or Scheie syndrome.

In a seventh aspect, the present invention features novel pharmaceutical compositions comprising  $\alpha$ -L-iduronidase useful for treating a disease caused all or in part by a deficiency in  $\alpha$ -L-iduronidase. Such compositions may be suitable for administration in a number of ways such as parenteral, topical, intranasal, inhalation or oral administration. Within the scope of this aspect are embodiments featuring nucleic

acid sequences encoding all or a part of an  $\alpha$ -L-iduronidase, which may be administered in vivo into cells, affected with an  $\alpha$ -L-iduronidase deficiency.

#### DESCRIPTION OF THE FIGURES

FIGURE 1 represents the nucleotide and deduced amino acid sequences of cDNA encoding  $\alpha$ -L-iduronidase (SEQ ID NOS:1 and 2). Nucleotides 1 through 6200 are provided. Amino acids are provided starting with the first methionine in the open reading frame.

FIGURE 2 represents the results from SDS-PAGE runs of eluate obtained according to the procedures as described below. The top panel shows the SDS-PAGE results of purified  $\alpha$ -L-iduronidase (3 micrograms) and contaminants from the production/purification scheme disclosed in Kakkis, *et al.*, *Protein Expr. Purif.* 5: 225-232 (1994). In the bottom panel, SDS-PAGE results of purified  $\alpha$ -L-iduronidase with contaminants from an unpublished prior production/purification process (U.S. Patent Application Serial Nos. 09/078,209 and 09/170,977) referred to as the Carson method in Lanes 2 (7.5 microgram  $\alpha$ -L-iduronidase) and Lane 3 (5.0 microgram  $\alpha$ -L-iduronidase) are compared to that of the production/purification process of the present invention referred to as the Galli Process (Lane 4 5 micrograms  $\alpha$ -L-iduronidase). Lane 1 contains the molecular weight marker. FIGURE 2 shows that the Galli production/purification method of the present invention yields a highly purified  $\alpha$ -L-iduronidase product with fewer contaminants in comparison with prior production/purification schemes.

FIGURE 3 demonstrates the  $\alpha$ -iduronidase production level over a 30-day period, during which time cells are switched at day 5 from a serum – containing medium to a serum-free medium.  $\alpha$ -Iduronidase production was characterized by: (1) absence of a need for adaptation when cells are switched from serum-containing to serum-free medium at 100200 (top and bottom panels) with an uninterrupted increase in productivity (top panel); (2) a high level of production in excess of 4 mg per liter (1000 per mL) in a protein-free medium (bottom panel); and (3) a boost in  $\alpha$ -iduronidase production with butyrate induction events (bottom panel).

FIGURE 4 demonstrates a decrease in liver volume during enzyme therapy in MPS I patients.

FIGURE 5 demonstrates urinary GAG excretion during enzyme therapy.

FIGURE 6 demonstrates elbow and knee extension in HAC002 during enzyme therapy.

FIGURE 7 demonstrates shoulder flexion to 104 weeks in four patients with the most restriction during enzyme therapy.

5       FIGURE 8 demonstrates improvement in sleep apnea before and after six weeks of therapy.

FIGURE 9 demonstrates the improvement in apneas and hypopneas during sleep with enzyme therapy in each individual patient.

10       FIGURE 10 demonstrates the improvement in pulmonary function tests before and after 12 and 52 weeks of enzyme therapy in one patient.

FIGURE 11 demonstrates increased height growth velocity with enzyme therapy.

FIGURE 12 shows the degree of contamination by Chinese Hamster Ovary Protein (CHOP) and degree of purity of  $\alpha$ -L-iduronidase, produced by (1) the Carson method, an unpublished prior production/purification process (U.S. Patent Application  
15       Serial Nos. 09/078,209 and 09/170,977 and (2) the Galli method, the production/purification process of the present invention. Thus, FIGURE 12 shows that  $\alpha$ -L-iduronidase produced and purified by the Galli method has a higher degree of purity and lower degree of CHOP contamination in comparison to that of the Carson method.

FIGURE 13 shows a comparison of  $\alpha$ -L-iduronidase produced by the Galli method  
20       versus the Carson method. On the left side of the Figure, results from a Western Blot show that the Galli material (left side, column 2) comprise fewer contaminating protein bands (between 48 kDa and 17 kDa) in comparison with the Carson material (left side, column 3). On the right side of the Figure, results from an SDS-PAGE silver stain show the absence of a band at the 62 kDa in the Galli material (column 2) in comparison to the  
25       presence of such a band in the Carson material (column 3).

#### DETAILED DESCRIPTION OF THE INVENTION

In one aspect, the present invention features a method to produce  $\alpha$ -L-iduronidase in amounts which enable using the enzyme therapeutically. In general, the method  
30       features transforming a suitable cell line with the cDNA encoding for all of  $\alpha$ -L-iduronidase or a biologically active fragment or mutein thereof. Those of skill in the art may prepare expression constructs other than those expressly described herein for optimal

production of  $\alpha$ -L-iduronidase in suitable cell lines transfected therewith. Moreover, skilled artisans may easily design fragments of cDNA encoding biologically active fragments and muteins of the naturally occurring  $\alpha$ -L-iduronidase which possess the same or similar biological activity to the naturally occurring full-length enzyme.

5 To create a recombinant source for  $\alpha$ -L-iduronidase, a large series of expression vectors may be constructed and tested for expression of a  $\alpha$ -L-iduronidase cDNA. Based on transient transfection experiments, as well as stable transfections, an expression construct may be identified that provides a particularly high level of expression. In one embodiment of the present invention, a Chinese hamster cell line 2.131 developed by  
10 transfection of the  $\alpha$ -L-iduronidase expression construct and selection for a high expression clone provides particularly high level expression. Such a Chinese hamster cell line according to this embodiment of the present invention may secrete about 5,000 to 7,000 fold more  $\alpha$ -L-iduronidase than normal. The  $\alpha$ -L-iduronidase produced thereby may be properly processed, taken up into cells with high affinity and is corrective for  $\alpha$ -L-  
15 iduronidase deficient cells, such as those from patients suffering from Hurler's Syndrome.

The method for producing  $\alpha$ -L-iduronidase in amounts that enable using the enzyme therapeutically features a production process specifically designed to mass produce commercial grade enzyme, wherein the quality of the enzyme has been deemed acceptable for administration to humans by regulatory authorities of various countries.  
20 The large scale production of commercial grade enzyme necessitates modifications of the cell culture scale, microcarrier systems, and purification scheme. In preferred embodiments, the cell culture scale is increased from 45 liters to 110 liters or more, with a change to continuous perfusion. The increase in scale is necessary to produce sufficient material for potential large scale production for long term patient use. According to  
25 preferred embodiments of such a process, microcarriers are used as a low cost scalable surface on which to grow adherent cells. In particularly preferred embodiments, such microcarriers are macroporous and are specifically composed of modified carbohydrates such as cellulose, e.g., Cytopore beads manufactured by Pharmacia. Macroporous cellulose microcarriers allow improved cell attachment and provide a larger surface area  
30 for attachment, which is expected to yield an increased cell density during the culture process. Higher cell densities are expected to increase productivity. In preferred embodiments, heparin-Sepharose and Sephacryl 200 columns are replaced with Blue-



Sepharose and Copper chelating columns to increase the capacity of the large scale purification process and to improve the purity of the product. In a particularly preferred embodiment, the copper chelating column is used to reduce Chinese hamster ovary cell protein contaminants to very low levels appropriate for large scale distribution. Using  
5   embodiments of the present method featuring modifications and induction described below, approximately 15 mg per liter of culture per day, or more at peak culturing density can be produced starting with a 110 liter culture system.

According to other preferred embodiments of the method for producing  $\alpha$ -L-iduronidase according to the present invention, a culture system is optimized. In a first  
10   embodiment, the culture pH is lowered to about 6.5 to 7.0, preferably to about 6.7-7.0 during the production process. One advantage of such a pH is to enhance accumulation of lysosomal enzymes that are more stable at acidic pH. In a second embodiment, as many as 2 to 3.5 culture volumes of the medium may be changed during each 24-hour period by continuous perfusion. One advantage of this procedure is to enhance the secretion rate of  
15   recombinant  $\alpha$ -L-iduronidase and to capture more active enzyme. In a third embodiment, oxygen saturation is optimized at about 40%. In a fourth embodiment, macroporous microcarriers with about 5% serum initially in the medium, are used to produce a cell mass followed by a rapid washout shift to a protein-free medium for production (FIGURE 3). In a fifth embodiment, a protein-free growth medium, such as a JRH Biosciences PF-  
20   CHO product, may be optimized to include supplemental amounts of one or more ingredients selected from the group consisting of: glutamate, aspartate, glycine, ribonucleosides and deoxyribonucleosides. In a sixth embodiment, as many as 2 to 3.5 culture volumes of the medium may be changed during each 24-hour period by continuous perfusion. Such an induction process may provide about a two-fold increase  
25   in production without significantly altering post-translational processing.

Particularly preferred embodiments of the method for producing  $\alpha$ -L-iduronidase according to the present invention feature one, more than one, or all of the optimizations described herein and may be employed as described in more detail below. The production method of the present invention may, therefore, provide a production culture process  
30   having the following features:

1.     A microcarrier based culture using macroporous microcarrier beads made of modified cellulose or an equivalent thereof is preferably used in large scale culture

flasks with overhead stirring or an equivalent thereof. Attachment of cells to these beads may be achieved by culture in a 5% fetal bovine serum may be added to DME/F12 1:1 or a protein-free medium modified with ingredients including ribonucleosides, deoxyribonucleosides, pyruvate, non-essential amino acids, and HEPES. After about 3-6 days in this medium, a washout procedure is begun in which protein-free medium replaces the serum-containing medium at an increasing perfusion rate dependent on the glucose content and culture condition. Subsequently, and throughout the entire remaining culture period, the cells are cultivated in a protein-free medium. The use of a protein-free medium in enzyme production is beneficial in reducing the exposure risk of bovine spongiform encephalopathy (BSE) and other infectious biologic agents such as viruses to patients being treated with the enzyme, wherein the risk of BSE or other harmful agents is dependent on the amount of potential serum exposure. In prior published studies, the carriers used to grow the cells were bovine gelatin microcarriers, used at 1 gram per liter or 100 times the product concentration. Leaching of 1% of the gelatin protein from the microcarriers would represent a relative 100% contamination and thereby contribute to the risk of BSE. Thus, new carriers are either dextran or cellulose-based and consist of carbohydrates, and not animal-derived materials.

FIGURE 3 shows that the cells are grown to a density in 5% serum containing medium and then switched without any adaptation to a protein-free medium. FIGURE 3 specifically shows that: 1) Cells survive and continue to produce iduronidase when shifted without adaptation. In contrast, other studies would suggest that adaptation to a protein-free medium is necessary. In the method of the present invention, enzyme production continues at levels comparable to serum containing medium. 2)  $\alpha$ -L-Iduronidase produced in a protein-free medium retains a level of production in excess of 4 mg per liter or 1,000 units per ml. 3)  $\alpha$ -L-Iduronidase produced in a protein-free medium has high uptake indicating that the shift in medium and, hence, a shift in carbohydrates being fed to cells, does not adversely affect the high uptake character of the enzyme. Eight lots of  $\alpha$ -L-iduronidase have been produced and released in this manner with an uptake half maximal value of less than 2nM in all lots.

2. The culture conditions are preferably maintained at a dissolved oxygen of 40% of air saturation at a pH of about 6.8-7.0 and at a temperature of about 35-37° C. This may be achieved using a control unit, monitoring unit and appropriate probes such as

those produced by Applikon® or Mettler®. However, skilled artisans will readily appreciate that this can easily be achieved by equivalent control systems produced by other manufacturers. An air saturation of about 40% results in improved  $\alpha$ -L-iduronidase secretion though up to 80%% air saturation may be used. However, further increases in oxygen to, for example, 90% air saturation, do not provide significantly enhanced secretion over 80% air saturation. The dissolved oxygen may be supplied by intermittent or continuous oxygen sparging using a 5 micron stainless steel or larger opening sparger, or equivalent thereof. A pH of about 6.8-7.0 is optimal for the accumulation of the  $\alpha$ -L-iduronidase enzyme. The enzyme is particularly unstable at pHs above about 7.0. Below a pH of about 6.7, the secretion rate may decrease, particularly below a pH of about 6.5. The culture is therefore maintained optimally between a pH of about 6.8-7.0 .

3. The production culture medium may be a modified form of the commercially available proprietary medium from JRH Biosciences called Excell PF CHO. This medium supports levels of secretion equivalent to that of serum using a cell line such as the 2.131 cell line. It may be preferably modified to include an acidic pH of about 6.8-7.0 ( $\pm 0.1$ ), and buffered with HEPES at 7.5 mM or 15 mM. The medium may contain 0.05 to 0.1% of Pluronic F-68 (BASF), a non-ionic surfactant or an equivalent thereof which features the advantage of protecting cells from shear forces associated with sparging. The medium may further contain a proprietary supplement that is important in increasing the productivity of the medium over other protein-free media that are presently available. Those skilled in the art will readily understand that the choice of culture medium may be optimized continually according to particular commercial embodiments available at particular points in time. Such changes encompass no more than routine experimentation and are intended to be within the scope of the present invention.

4. The production medium may be analyzed using an amino acid analyzer comparing spent medium with starting medium. Such analyses have demonstrated that the 2.131 cell line depletes a standard PF CHO medium of glycine, glutamate and aspartate to a level of around 10% of the starting concentration. Supplementation of these amino acids to higher levels may result in enhanced culture density and productivity that may lead to a 2-3 fold higher production than at baseline. Skilled artisans will appreciate that other cell lines within the scope of the present invention may be equally useful for producing  $\alpha$ -L-iduronidase according to the present method. Hence, more or less

supplemental nutrients may be required to optimize the medium. Such optimizations are intended to be within the scope of the present invention and may be practiced without undue experimentation.

5           5.       The medium may be supplemented with the four ribonucleosides and four deoxyribonucleosides each at about 10 mg/liter to support the dihydrofolate reductase deficient cell line 2.131. Skilled artisans will appreciate that other cell lines within the scope of the present invention may be equally useful for producing  $\alpha$ -L-iduronidase according to the present method. Hence, more or less ribonucleosides and deoxyribonucleosides may be required to optimize the medium, and alternative sources of purines and pyrimidines for nucleic acid synthesis may be used such as hypoxanthine and thymidine. Such optimizations are intended within the scope of the present invention and may be practiced without undue experimentation.

10           6.       After reaching confluence at about 3-6 days of culture, an increasing rate of continuous perfusion is initiated. A change of medium may be accomplished, for example, using a slant feed tube constructed and positioned to allow the uptake of medium without removal of the microcarriers even while the culture is stirred. By pumping out medium through the slant feed tube, microcarriers settle within the body of the tube inside the culture and are not removed from the culture during the change on medium. In this manner, the microcarriers with the cell mass are separated from supernatant containing the enzyme.

15           7.       The rapid and frequent turnover of the medium has been shown by productivity studies to result in improved overall collection of enzyme from the cell culture. Less turnover of medium results in less total production of enzyme on a daily basis. Using the perfusion of 2-3.5 culture volumes per day, the cells may be maintained in excellent condition with high degrees of viability and a high level of productivity.

20           8.       Production of  $\alpha$ -L-iduronidase may be enhanced by the use of sodium butyrate induction of gene expression (FIGURE 3). Twenty lots of  $\alpha$ -L-iduronidase were produced using butyrate induction at 2nM concentration with 2/3 washout every 12 hours after induction and reinduction every 48 hours for a 21-day production period. In Figure 3, the vertical arrows at the bottom indicate butyrate induction events. Each induction triggered a boost in  $\alpha$ -L-iduronidase concentration in the medium.

Systematic studies of a 2.131 cell line demonstrated that about 2 mM butyrate can be applied and result in about a two-fold or greater induction of enzyme production with minimal effects on carbohydrate processing. Lower levels of butyrate have not been shown to induce as well, and substantially higher levels may result in higher induction, but declining affinity of the produced enzyme for cells from patients suffering from  $\alpha$ -L-iduronidase deficiency. Butyrate induction performed *in vitro* at 2mM for 24 hours or 5 mM, a more commonly used concentration resulted in uptakes in excess of 3 nM or 40 U/ml, or an average of three times the value observed in production lots. In addition, commonly used times of 24 hours or more and concentration of 5 mM were toxic to  $\alpha$ -L-iduronidase producing cells and resulted in detachment and loss of cell mass.

Results suggest that two-fold or greater induction results in less processing of the carbohydrates and less phosphate addition to the enzyme, as well as increasing toxicity. With respect to carbohydrate processing and the addition of phosphate groups, the importance of mannose-6-phosphate in enzyme replacement therapy is demonstrated by the observations that removal of the phosphate of two lysosomal enzymes, glucosidase and galactosamine 4-sulfatase leads to decreased uptake (Van der Ploeg, *et al.*, *J. Clin. Invest.* 87: 513-518 (1991); Crawley, *et al.*, *J. Clin. Invest.* 97: 1864-1873 (1996)). In addition, enzyme with low phosphate (Van Hove, *et al.*, *Proc. Natl. Acad. Sci. USA* 93: 65-70 (1996) requires 1,000 units per ml for uptake experiments (nearly 100 times used for iduronidase) and effective doses in animal models require 14 mg/kg, or 28 times the dose used with high phosphate containing iduronidase (Kikuchi, *et al.*, *J. Clin. Invest.* 101: 827-833 (1998)).

One particularly preferred aspect of the invention method uses 2 mM butyrate addition every 48 hours to the culture system. This embodiment results in about a two-fold induction of enzyme production using this method without significant effect on the uptake affinity of the enzyme (K-uptake of less than 30 U/ml or 2.0 mM).

In a second aspect, the present invention provides a transfected cell line, which possesses the unique ability to produce  $\alpha$ -L-iduronidase in amounts, which enable using the enzyme therapeutically. In preferred embodiments, the present invention features a recombinant Chinese hamster ovary cell line such as the 2.131 cell line that stably and reliably produces amounts of  $\alpha$ -L-iduronidase. In preferred embodiments, the cell line may contain more than 1 copy of an expression construct comprising a CMV promoter, a

C $\alpha$  intron, a human  $\alpha$ -L-iduronidase cDNA, and a bovine growth hormone polyadenylation sequence. In even more preferred embodiments, the cell line expresses  $\alpha$ -L-iduronidase at amounts of at least about 20-40 micrograms per  $10^7$  cells per day in a properly processed, high uptake form appropriate for enzyme replacement therapy.

5 According to preferred embodiments of this aspect of the invention, the transfected cell line adapted to produce  $\alpha$ -L-iduronidase in amounts which enable using the enzyme therapeutically, possesses one or more of the following features:

1. The cell line of preferred embodiments is derived from a parent cell line wherein the cells are passaged in culture until they have acquired a smaller size and more  
10 rapid growth rate and until they readily attach to substrates.

2. The cell line of preferred embodiments is transfected with an expression vector containing the cytomegalovirus promoter/enhancer element, a 5' intron consisting of the murine C $\alpha$  intron between exons 2 and 3, a human cDNA of about 2.2 kb in length, and a 3' bovine growth hormone polyadenylation site. This expression vector may be  
15 transfected at, for example, a 50 to 1 ratio with any appropriate common selection vector such as pSV2NEO. The selection vector pSV2NEO in turn confers G418 resistance on successfully transfected cells. In particularly preferred embodiments, a ratio of about 50 to 1 is used since this ratio enhances the acquisition of multiple copy number inserts. According to one embodiment wherein the Chinese hamster ovary cell line 2.131 is  
20 provided, there is at least 1 copy of the expression vector for  $\alpha$ -L-iduronidase. Such a cell line has demonstrated the ability to produce large quantities of human  $\alpha$ -L-iduronidase (minimum 20 micrograms per 10 million cells per day). Particularly preferred embodiments such as the 2.131 cell line possess the ability to produce properly processed enzyme that contains N-linked oligosaccharides containing high mannose chains modified  
25 with phosphate at the 6 position in sufficient quantity to produce an enzyme with high affinity (K<sub>u</sub>-uptake of less than 3 nM).

3. The enzyme produced from the cell lines of the present invention such as a Chinese hamster ovary cell line 2.131 is rapidly assimilated into cells, eliminates glycosaminoglycan storage and has a half-life of about 5 days in cells from patients  
30 suffering from  $\alpha$ -L-iduronidase deficiency.

4. The cell line of preferred embodiments such as a 2.131 cell line adapts to large scale culture and stably produces human  $\alpha$ -L-iduronidase under these conditions.

The cells of preferred embodiments are able to grow and secrete  $\alpha$ -L-iduronidase at the acid pH of about 6.6 to 7.0 at which enhanced accumulation of  $\alpha$ -L-iduronidase can occur.

5        5.        Particularly preferred embodiments of the cell line according to the invention, such as a 2.131 cell line are able to secrete human  $\alpha$ -L-iduronidase at levels exceeding 2,000 units per ml (8 micrograms per ml) harvested twice per day or exceeding 15 mg per liter of culture per day using a specially formulated protein-free medium.

10        In a third aspect, the present invention provides novel vectors suitable to produce  $\alpha$ -L-iduronidase in amounts which enable using the enzyme therapeutically. The production of adequate quantities of recombinant  $\alpha$ -L-iduronidase is a critical prerequisite for studies on the structure of the enzyme as well as for enzyme replacement therapy. The cell lines according to the present invention permit the production of significant quantities of recombinant  $\alpha$ -L-iduronidase that is appropriately processed for uptake. Overexpression in Chinese hamster ovary (CHO) cells has been described for three other  
15        lysosomal enzymes,  $\alpha$ -galactosidase (Ioannou, *et al.*, *J Cell. Biol.* 119:1137-1150 (1992)), iduronate 2-sulfatase (Bielicki, *et al.*, *Biochem. J.* 289: 241-246 (1993)), and N-acetylgalactosamine 4 -sulfatase (Amson, *et al.*, *Biochem. J.* 284:789-794 (1992)), using a variety of promoters and, in one case, amplification. The present invention features a dihydrofolate reductase-deficient CHO cell line, but according to preferred embodiments  
20        of the invention amplification is unnecessary. Additionally, the present invention provides a high level of expression of the human  $\alpha$ -L-iduronidase using the CMV immediate early gene promoter/enhancer.

25        The present invention features in preferred embodiments, an expression vector comprising a cytomegalovirus promoter/enhancer element, a 5' intron consisting of the murine C $\alpha$  intron derived from the murine long chain immunoglobulin C $\alpha$  gene between exons 2 and 3, a human cDNA of about 2.2 kb in length, and a 3' bovine growth hormone polyadenylation site. This expression vector may be transfected at, for example, a 50 to 1 ratio with any appropriate common selection vector such as pSV2NEO. The selection vector such as pSV2NEO in turn confers G418 resistance on successfully transfected  
30        cells. In particularly preferred embodiments, a ratio of about 50 to 1 expression vector to selection vector is used since this ratio enhances the acquisition of multiple copy number inserts. According to one embodiment wherein the Chinese hamster ovary cell line 2.131

is provided, there are approximately 10 copies of the expression vector for  $\alpha$ -L-iduronidase. Such an expression construct has demonstrated the ability to produce large quantities of human  $\alpha$ -L-iduronidase (minimum 20 micrograms per 10 million cells per day) in a suitable cell line such as a Chinese hamster ovary cell line 2.131.

5 In a fourth aspect, the present invention provides novel  $\alpha$ -L-iduronidase produced in accordance with the methods of the present invention and thereby present in amounts that enable using the enzyme therapeutically. The methods of the present invention produce a substantially pure  $\alpha$ -L-iduronidase that is properly processed and in high uptake form, appropriate for enzyme replacement therapy and effective in therapy *in vivo*.

10 The specific activity of the  $\alpha$ -L-iduronidase according to the present invention is in excess of about 200,000 units per milligram protein. Preferably, it is in excess of about 240,000 units per milligram protein using the original assay methods for activity and protein concentration. A novel validated assay for the same enzyme with units expressed as micromoles per min demonstrates an activity of 100 units/ml (range of 70-130) and a  
15 protein concentration by absorbance at 280 nM of 0.7 mg/ml (0.6-0.8) with an average specific activity of 143 units per mg. The molecular weight of the full length  $\alpha$ -L-iduronidase of the present invention is about 82,000 daltons comprising about 70,000 daltons of amino acids and 12,000 daltons of carbohydrates. The recombinant enzyme of the present invention is endocytosed even more efficiently than has been previously  
20 reported for a partially purified preparation of urinary enzyme. The recombinant enzyme according to the present invention is effective in reducing the accumulation of radioactive S-labeled GAG in  $\alpha$ -L-iduronidase-deficient fibroblasts, indicating that it is transported to lysosomes, the site of GAG storage. The remarkably low concentration of  $\alpha$ -L-iduronidase needed for such correction (half-maximal correction at 0.7 pM) may be very  
25 important for the success of enzyme replacement therapy.

The human cDNA of  $\alpha$ -L-iduronidase predicts a protein of 653 amino acids and an expected molecular weight of 70,000 daltons after signal peptide cleavage. Amino acid sequencing reveals alanine 26 at the N-terminus giving an expected protein of 629 amino acids. Human recombinant  $\alpha$ -L-iduronidase has a Histidine at position 8 of the  
30 mature protein. The predicted protein sequence comprises six potential N-linked oligosaccharide modification sites. All of these may be modified in the recombinant protein. The third and sixth sites have been demonstrated to contain one or more

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mannose 6-phosphate residues responsible for high affinity uptake into cells. The following peptide corresponds to Amino Acids 26-45 of Human Recombinant  $\alpha$ -L-iduronidase with an N-terminus alanine and the following sequence:

ala-glu-ala-pro-his-leu-val-his-val-asp-ala-ala-arg-ala-leu-trp-pro-leu-arg-arg

5 (part of SEQ ID NO:2)

The overexpression of the  $\alpha$ -L-iduronidase of the present invention does not result in generalized secretion of other lysosomal enzymes that are dependent on mannose-6-P targeting. The secreted recombinant  $\alpha$ -L-iduronidase is similar to normal secreted enzyme in many respects. Its molecular size, found in various determinations to be 77,  
10 82, 84, and 89 kDa, is comparable to 87 kDa, found for urinary corrective factor (Barton *et al.*, *J. Biol. Chem.* 246: 7773-7779 (1971)), and to 76 kDa and 82 kDa, found for enzyme secreted by cultured human fibroblasts (Myerowitz, *et al.*, *J. Biol. Chem.* 256: 3044-3048 (1991); Taylor, *et al.*, *Biochem. J* 274:263-268 (1991)). The differences within and between the studies are attributed to imprecision of the measurements. The  
15 pattern of intracellular processing of the recombinant enzyme, a slow decrease in molecular size and the eventual appearance of an additional band smaller by 9 kDa is the same as for the human fibroblast enzyme. This faster band arises by proteolytic cleavage of 80 N-terminal amino acids.

In a fifth aspect, the present invention features a novel method to purify  $\alpha$ -L-iduronidase. The U.S. Food and Drug Administration has issued recommendations for  
20 assembling chemical and technological data currently considered appropriate for an enzyme preparation, including guidelines regarding the purity of enzyme preparations (Enzyme Preparations: Chemistry Recommendations for Food Additive and GRAS [Generally Recommended As Safe] Affirmation Petitions, Version 1.1, January 23, 1993;  
25 U.S. Food and Drug Administration, Center For Food Safety and Applied Nutrition, Office of Premarket Approval, Chemistry Review Branch). Various studies have shown that impurities, such as anticomplement activity, in protein preparations, including immunoglobulin preparations, may be associated with the development of allergic and anaphylactic reactions (Lundblad, *et al.*, *Rev. Infect. Dis.* 8 (Suppl. 4):S382-90 (1986);  
30 Scheiermann and Kuwert, *Dev. Biol. Stand.* 44:165-171 (1979)). Furthermore, impurities may be associated with unwanted biological activities and interference with desired therapeutic effects. Thus, enhanced purity of protein preparations would contribute to

greater efficacy of the therapeutic protein (Ueshima, *et al.*, *J. Clin. Hosp. Pharm.* 10(2): 193-202 (1985); Ehrlich, *et al.*, *Clin. Chem.* 34(9): 1681-8 (1988)).

The relationship between enzyme purity and immunogenicity is demonstrated in Studies 1 (Example 5) and 2 (Example 6). Two types of immune reactions, urticaria and complement activation (indicated by laboratory analysis), were documented during enzyme infusion and may be associated with enzyme therapy. In the Phase I study (Example 5), the purity of recombinant human  $\alpha$ -L-iduronidase was between 96% to 98%. In the Phase III study (Example 6), recombinant human  $\alpha$ -L-iduronidase was purified to greater than 99%. Figures 12 and 13 compare the degree of contamination by the other proteins, such as Chinese Hamster Ovary Protein, and the purity of the recombinant human  $\alpha$ -L-iduronidase produced by the previous Carson and current Galli methods. The results show that the recombinant human  $\alpha$ -L-iduronidase purified according to the Galli method has fewer protein contaminants than enzyme produced by the Carson method. In the Phase I study using enzyme purified to 96-98%, five patients developed urticaria and evidence of complement activation was observed in four patients. In the Phase III study using enzyme purified to greater than 99%, none of the enzyme-treated patients developed urticaria. Although all enzyme – treated patients seroconverted in immunogenicity testing for IgG, seroconversion did not result in increased infusion-associated reactions or other adverse events. In patients tested for IgE, results were negative. The relationship between purity and immunogenicity is even more evident in the animal studies described in Example 3, wherein the purity of the recombinant human  $\alpha$ -L-iduronidase was equal or less than or about 95%. In the animal studies, all MPS I dogs and most MPS I cats receiving enzyme treatment developed antibodies, including IgG antibodies of the complement-activating type, a phenomenon observed in 13 % of alglucerase-treated Gaucher patients. One MPS I dog also developed proteinuria thought to be related to immune complex disease. These studies suggest that an increased level of enzyme purity is associated with a lower frequency of immune-related adverse side effects and hence with greater safety and efficacy of enzyme therapy.

In preferred embodiments, the present invention features a method to purify recombinant  $\alpha$ -L-iduronidase that has been optimized to produce a rapid and efficient purification with validatable chromatography resins and easy load, wash and elute operation. The method of purifying  $\alpha$ -L-iduronidase of the present invention involves a

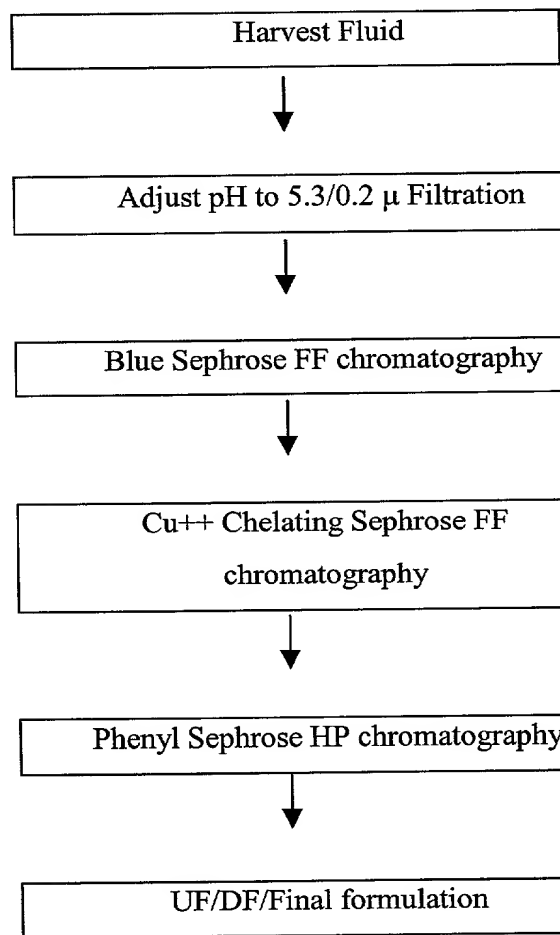
series of column chromatography steps, which allow the high yield purification of enzyme from protein-free production medium. Specifically, Concanavalin A-Sepharose, Heparin-Sepharose and Sephacryl 200 columns were replaced with Blue-Sepharose and Copper chelating columns to increase the capacity of a large-scale purification process, to reduce leachables and to improve the purity of the product. Concanavalin A lectin is often used to bind enzyme in an initial purification step in the prior published study, and is a protein lectin derived from plants. Concanavalin A is known to leach from columns and contaminate lysosomal enzyme preparations. Such leaching could cause activation of T cells in treated patients and hence is deemed inappropriate for human administration (Furbish, *et al.*, *Proc. Natl. Acad. Sci. USA* 74: 3560-3563 (1977)). Thus, the use of Concanavalin A is avoided in the present purification scheme. In a prior study, the human liver  $\alpha$ -L-iduronidase could not be recovered from phenyl columns without high concentrations of detergent (1% Triton X100) denaturation. Hence, a phenyl column was not used in a published purification scheme of this enzyme (Clements, *et al.*, *Eur. J. Biochem.* 152: 21-28 (1985)). The endogenous human liver enzyme is highly modified within the lysosomes by hydrolases, which remove sialic acid and phosphate residues, and proteases, which nick the enzyme. In contrast, the overexpression of recombinant  $\alpha$ -L-iduronidase causes 50% of the enzyme to be secreted rather than transported to the lysosome (Zhao, *et al.*, *J. Biol. Chem.* 272: 22758-22765 (1997)). Hence, recombinant iduronidase will have a full array of sialic acid and phosphate residues, which lead to a higher degree of water solubility and lower affinity to the phenyl column. The increased hydrophilicity allows the enzyme to be eluted under non-denaturing conditions using the low salt solutions of around 150-700 mM NaCl. This feature of the recombinant enzyme allows it to be purified in large scale without the use of detergents.

Recombinant  $\alpha$ -L-iduronidase over-expressed in a Chinese Hamster Ovary (CHO) cell line, has been purified to near homogeneity following a 3-step column chromatography process. The first column involves an affinity chromatography step using Blue Sepharose 6 FF. The Blue Sepharose 6 FF eluate is then further purified by another affinity chromatography step using  $\text{Cu}^{++}$  Chelating Sepharose FF. The final polish of the highly purified enzyme is achieved by hydrophobic interaction chromatography using Phenyl Sepharose High Performance (HP). The over-all yield ranges from 45 to 55 percent and the purity of the final product is > 99%. The process is

robust, reproducible, and scalable for large-scale manufacturing. The purified enzyme has been characterized with respect to its enzymatic activity using a fluorescence-based substrate, and its functional uptake by fibroblast cells. The enzyme has also been characterized for substrate specificity, carbohydrate profiles, and isoelectric focusing (IEF) profiles.

Particularly preferred embodiments of the method for purifying  $\alpha$ -L-iduronidase according to the present invention feature more than one or all of the optimizations according to the following particular embodiments. The purification method of the present invention may therefore provide a purified  $\alpha$ -L-iduronidase having the characteristics described herein.

#### Outline of the $\alpha$ -L-Iduronidase Purification Process



2. Blue Sepharose FF chromatography: This affinity chromatography step serves to capture iduronidase to reduce the volume and to purify iduronidase by approximately seven to ten fold.

Equilibration buffer: 10 mM NaPO<sub>4</sub>, pH 5.3

10 Elution buffer: 0.8 M NaCl, 10 mM NaPO<sub>4</sub>, pH 5.3

Fold of purification: 7-10

### 3. $\text{Cu}^{++}$ Chelating Sepharose FF chromatography: The $\text{Cu}^{++}$ Chelating

15 affinity chromatography step is very effective for removing some contaminating CHO  
proteins. The inclusion of 10% glycerol in all the buffers seems to be crucial for the  
quantitative recovery of iduronidase.

Equilibration buffer: 1 M NaCl, 25 mM NaAc, pH 6.0, 10% Glycerol

20 Wash buffer: 1 M NaCl, 25 mM NaAc, pH 4.0, 10% Glycerol

Elution buffer: 1 M NaCl, 25 mM NaAc, pH 3.7, 10% Glycerol

Regeneration buffer: 1 M NaCl, 50 mM EDTA, pH 8.0

Fold of purification: 2-5

Yield: 80%

25            4.        Phenyl Sephrose HP chromatography: Phenyl Sephrose is used as the last step to further purify the product as well as to reduce residual leached Cibacron blue dye and  $\text{Cu}^{++}$  ion carried over from previous columns.

Loading capacity: 1 mg/ml

Equilibration buffer: 2 M NaCl, 10 mM NaPO<sub>4</sub>, pH 5.7

30 Wash buffer: 1.5 M NaCl, 10 mM NaPO<sub>4</sub>, pH 5.7

Elution buffer: 0.7 M NaCl, 10 mM NaPO<sub>4</sub>, pH 5.7

Regeneration buffer: 0 M NaCl, 10 mM NaPO<sub>4</sub>, pH 5.7  
Fold of purification: 1.5  
Yield: 90%

5 5. Ultrafiltration (UF)/Diafiltration (DF)/Final formulation: The purified iduronidase is concentrated and diafiltered to a final concentration of 1 mg/ml in formulation buffer (150 mM NaCl, 100 mM NaPO<sub>4</sub>, pH 5.8) using a tangential flow filtration (TFF) system (e.g. Sartocoon Slice from Sartorius). The enzyme is then sterilized by filtering through a 0.2-micron filter (e.g., cellulose acetate or polysulfone) and filled into sterile vials.

10 6. Characterization of Purified Iduronidase: Analysis of enzyme purity using SDS-PAGE stained with Coomassie Blue or Silver and Western blot analysis. Analysis of enzymatic activity using 4MU-sulfate as substrate. Analysis of functional uptake using fibroblast cell assay. Analysis of carbohydrates by FACE. Analysis of IEF profiles.

15 Enzyme purified in this manner has been shown to contain mannose-6-phosphate residues of sufficient quantity at positions 3 and 6 of the N-linked sugars to give the enzyme uptake affinity of less than 30 units per ml (less than 2 nM) enzyme. The enzyme is substantially corrective for glycosaminoglycan storage disorders caused by iduronidase deficiency and has a half-life inside cells of approximately 5 days.

20 Prior  $\alpha$ -L-iduronidase purification schemes (Kakkis, *et al.*, *Protein Expr. Purif.* 5: 225-232 (1994); Kakkis, *et al.*, *Biochem. Mol. Med.* 58: 156-167 (1996); U.S. Patent Application Nos. 09/078,209 and 09/170,977) produced degrees of purity between 90% and less than 99%, which is not optimal for long-term human administration (See FIGURE 12). (These and all other U.S. patents herein are specifically incorporated herein by reference in their entirety.) Treatment with human recombinant  $\alpha$ -L-iduronidase with  
25 a minimum purity of 97% was associated with some clinical reactions, specifically hives in 5 patients, and complement activation in 4 patients. All patients demonstrated a reaction to a protein that is a trace contaminant to the  $\alpha$ -L-iduronidase. (FIGURE 2) Because this protein exists in both the final product and in the serum-free blank CHO cell line supernatant, the extraneous protein most likely originates from the CHO cell. The  
30 common proteins that appear to be activating the clinical allergic response are approximately 60kDaltons and 50kDaltons respectively, which are too small to be recombinant human iduronidase. Four patients developed an immune reaction to  $\alpha$ -L-

iduronidase at least transiently as well as to the Chinese hamster ovary cell host proteins. It is clear that even though the enzyme used to treat patients is highly purified, the degree of purification is important in reducing the immune response to contaminants. FIGURE 2 (SDS-PAGE), FIGURE 12 (CHOP assay), and FIGURE 13 (Western Blot, Silver Stain) demonstrate that  $\alpha$ -L-iduronidase produced and purified by the production/purification scheme of the present invention has a higher degree of purity and lower degree of CHOP contamination in comparison to that of prior methods of production/purification. Thus, a greater than 97% purity is adequate for patient use, higher levels of purity are desirable and preferable. As shown in FIGURE 12, the optimized purification scheme described above achieves a degree of purity that is greater than 99% and importantly reduces Chinese hamster ovary cell host proteins to less than 1 percent, as determined by the Chinese Hamster Ovary Protein (CHOP) assay.

In a sixth aspect, the present invention features novel methods of treating diseases caused all or in part by a deficiency in  $\alpha$ -L-iduronidase. Recombinant  $\alpha$ -L-iduronidase provides enzyme replacement therapy in a canine model of MPS 1. This canine model is deficient in  $\alpha$ -L-iduronidase due to a genetic mutation and is similar to human MPS 1. Purified, properly processed  $\alpha$ -L-iduronidase was administered intravenously to 11 dogs. In those dogs treated with weekly doses of 25,000 to 125,000 units per kg for 0.5, 3, 6 or 13 months, the enzyme was taken up in a variety of tissues and decreased the lysosomal storage in many tissues. The long term treatment of the disease was associated with clinical improvement in demeanor, joint stiffness, coat and growth. Higher doses of therapy (125,000 units per kg per week) result in better efficacy, including normalization of urinary GAG excretion in addition to more rapid clinical improvement in demeanor, joint stiffness and coat.

Enzyme therapy at even small doses of 25,000 units (0.1 mg/kg/wk) resulted in significant enzyme distribution to some tissues and decreases in GAG storage. If continued for over 1 year, some clinical effects were evident in terms of increased activity, size and overall appearance of health. The therapy at this dose did not improve other tissues that are important sites for disease in this entity such as cartilage and brain. Higher doses of 125,000 units (0.5 mg/kg) given 5 times over two weeks demonstrate that improved tissue penetration can be achieved, and a therapeutic effect at the tissue level was accomplished in as little as 2 weeks. Studies at this increased dose have been

completed in two dogs for 15 months. These MPS I dogs are showing significant clinical improvement and substantial decreases in urinary GAG excretion into the near normal range. Other than an immune reaction controlled by altered administration techniques, the enzyme therapy has not shown significant clinical or biochemical toxicity. Enzyme  
5 therapy at this higher weekly dose is effective at improving some clinical features of MPS I and decreasing storage without significant toxicity.

In a seventh aspect, the present invention features novel pharmaceutical compositions comprising human  $\alpha$ -L-iduronidase useful for treating a deficiency in  $\alpha$ -L-iduronidase. The recombinant enzyme may be administered in a number of ways such as  
10 parenteral, topical, intranasal, inhalation or oral administration. Another aspect of the invention is to provide for the administration of the enzyme by formulating it with a pharmaceutically acceptable carrier, which may be solid, semi-solid, liquid, or an ingestible capsule. Examples of pharmaceutical compositions include tablets, drops such as nasal drops, compositions for topical application such as ointments, jellies, creams and  
15 suspensions, aerosols for inhalation, nasal spray, and liposomes. Usually the recombinant enzyme comprises between 0.01 and 99% or between 0.01 and 99% by weight of the composition, for example, between 0.01 and 20% for compositions intended for injection and between 0.1 and 50% for compositions intended for oral administration.

To produce pharmaceutical compositions in this form of dosage units for oral  
20 application containing a therapeutic enzyme, the enzyme may be mixed with a solid, pulverulent carrier, for example lactose, saccharose, sorbitol, mannitol, a starch such as potato starch, corn starch, amylopectin, laminaria powder or citrus pulp powder, a cellulose derivative or gelatin and also may include lubricants such as magnesium or calcium stearate or a Carbowax® or other polyethylene glycol waxes and compressed to  
25 form tablets or cores for dragees. If dragees are required, the cores may be coated for example with concentrated sugar solutions which may contain gum arabic, talc and/or titanium dioxide, or alternatively with a film forming agent dissolved in easily volatile organic solvents or mixtures of organic solvents. Dyestuffs can be added to these coatings, for example, to distinguish between different contents of active substance. For  
30 the composition of soft gelatin capsules consisting of gelatin and, for example, glycerol as a plasticizer, or similar closed capsules, the active substance may be admixed with a Carbowax® or a suitable oil, *e.g.*, sesame oil, olive oil, or arachis oil. Hard gelatin



capsules may contain granulates of the active substance with solid, pulverulent carriers such as lactose, saccharose, sorbitol, mannitol, starches such as potato starch, corn starch or amylopectin, cellulose derivatives or gelatin, and may also include magnesium stearate or stearic acid as lubricants.

5           Therapeutic enzymes of the subject invention may also be administered parenterally such as by subcutaneous, intramuscular or intravenous injection or by sustained release subcutaneous implant. In subcutaneous, intramuscular and intravenous injection, the therapeutic enzyme (the active ingredient) may be dissolved or dispersed in a liquid carrier vehicle. For parenteral administration, the active material may be suitably  
10       admixed with an acceptable vehicle, preferably of the vegetable oil variety such as peanut oil, cottonseed oil and the like. Other parenteral vehicles such as organic compositions using solketal, glycerol, formal, and aqueous parenteral formulations may also be used.

          For parenteral application by injection, compositions may comprise an aqueous solution of a water soluble pharmaceutically acceptable salt of the active acids according  
15       to the invention, desirably in a concentration of 0.01-10%, and optionally also a stabilizing agent and/or buffer substances in aqueous solution. Dosage units of the solution may advantageously be enclosed in ampules.

          When therapeutic enzymes are administered in the form of a subcutaneous implant, the compound is suspended or dissolved in a slowly dispersed material known to  
20       those skilled in the art, or administered in a device which slowly releases the active material through the use of a constant driving force such as an osmotic pump. In such cases, administration over an extended period of time is possible.

          For topical application, the pharmaceutical compositions are suitably in the form of an ointment, gel, suspension, cream or the like. The amount of active substance may  
25       vary, for example, between 0.05- 20% by weight of the active substance. Such pharmaceutical compositions for topical application may be prepared in known manner by mixing the active substance with known carrier materials such as isopropanol, glycerol, paraffin, stearyl alcohol, polyethylene glycol, etc. The pharmaceutically acceptable carrier may also include a known chemical absorption promoter. Examples of absorption  
30       promoters are, *e.g.*, dimethylacetamide (U.S. Patent No. 3,472,931), trichloro ethanol or trifluoroethanol (U.S. Patent No. 3,891,757), certain alcohols and mixtures thereof (British Patent No. 1,001,949). A carrier material for topical application to unbroken skin

is also described in the British patent specification No. 1,464,975, which discloses a carrier material consisting of a solvent comprising 40-70% (v/v) isopropanol and 0-60% (v/v) glycerol, the balance, if any, being an inert constituent of a diluent not exceeding 40% of the total volume of solvent.

5           The dosage at which the therapeutic enzyme containing pharmaceutical compositions are administered may vary within a wide range and will depend on various factors such as the severity of the disease, the age of the patient, etc., and may have to be individually adjusted. A possible range for the amount of therapeutic enzyme which may be administered per day is about 0.1 mg to about 2000 mg or about 1 mg to about 2000  
10   mg.

          The pharmaceutical compositions containing the therapeutic enzyme may suitably be formulated so that they provide doses within these ranges, either as single dosage units or as multiple dosage units. In addition to containing a therapeutic enzyme (or therapeutic enzymes), the subject formulations may contain one or more substrates or cofactors for  
15   the reaction catalyzed by the therapeutic enzyme in the compositions. Therapeutic enzymes containing compositions may also contain more than one therapeutic enzyme.

          The recombinant enzyme employed in the subject methods and compositions may also be administered by means of transforming patient cells with nucleic acids encoding the recombinant  $\alpha$ -L-iduronidase. The nucleic acid sequence so encoded may be  
20   incorporated into a vector for transformation into cells of the subject to be treated. Preferred embodiments of such vectors are described herein. The vector may be designed so as to integrate into the chromosomes of the subject, *e.g.*, retroviral vectors, or to replicate autonomously in the host cells. Vectors containing encoding  $\alpha$ -L-iduronidase nucleotide sequences may be designed so as to provide for continuous or regulated  
25   expression of the enzyme. Additionally, the genetic vector encoding the enzyme may be designed so as to stably integrate into the cell genome or to only be present transiently. The general methodology of conventional genetic therapy may be applied to polynucleotide sequences encoding  $\alpha$ -L-iduronidase. Conventional genetic therapy techniques have been extensively reviewed. (Friedman, *Science* 244:1275-1281 (1989);  
30   Ledley, J. *Inherit. Metab. Dis.* 13:587-616 (1990); Tososhev, *et al.*, *Curr Opinions Biotech.* 1:55-61 (1990)).

A particularly preferred method of administering the recombinant enzyme is intravenously. A particularly preferred composition comprises recombinant  $\alpha$ -L-iduronidase, normal saline, phosphate buffer to maintain the pH at about 5.8 and human albumin. These ingredients may be provided in the following amounts:

$\alpha$ -L-iduronidase	0.05-0.2 mg/mL or 12,500-50,000 units per mL
Sodium chloride solution	150 mM in an IV bag, 50-250 cc total volume
Sodium phosphate buffer	10-50 mM, pH 5.8
Human albumin	1 mg/mL

Composition of Recombinant Human  $\alpha$ -L-Iduronidase (rhIDU,  
Aldurazyme™) Drug Product

Name of Ingredient	Concentration	Composition per vial	Function
rhIDU	100 U/mL (Range: 80 – 150 U/mL)	3.07 mg	Active ingredient
NaCl	150 mM	46.5 mg	Tonicity Modifier
Sodium Phosphate monobasic	92 mM	67.3 mg	Buffer
Sodium Phosphate dibasic	8 mM	11.3 mg	Buffer
Polysorbate 80	10 $\mu$ g/mL	0.05 mg	Stabilizer

The proposed commercial formulation for Aldurazyme™ is 100 Units/mL (approximately 0.58 mg/mL) for recombinant human  $\alpha$ -L-Iduronidase (rhIDU), 100 mM sodium phosphate, 150 mM sodium chloride, and 10  $\mu$ M/mL polysorbate 80, pH of 5.8. The Phase I study formula was identical to the Phase III study formula and proposed commercial formulation with the exception that polysorbate 80 was added as a stabilizer in the Phase III and commercial formula. This commercial formulation was also used in Good Laboratory Practice (GLP) toxicology studies.

Polysorbate 80, at a concentration of 10  $\mu$ M/mL was added to the formulation to act as a stabilizer. The change was implemented when the rhIDU production process was scaled up and prompted by the observation of a fine precipitate in the vial drug product and coincided with the change from polypropylene vials to glass vials. Formulation studies have demonstrated that polysorbate-20 (10  $\mu$ M/mL) and polysorbate-80 (5  $\mu$ M/mL) both minimized the formation of precipitates in vial Aldurazyme™ even after forced agitation. The concentration of polysorbate-20 or polysorbate-80 needed to minimize the formation of precipitates was 5  $\mu$ M/mL for polysorbate-80 and 10  $\mu$ M/mL for polysorbate-20. Preliminary data demonstrated that Aldurazyme™, when formulated with polysorbate 80 at 10  $\mu$ M/mL, retained activity when stored at 2-8°C. Polysorbate 80 was chosen over polysorbate 20 because it performed slightly better in preventing precipitate formation and it is more commonly used in marketed pharmaceutical product formulations. Polysorbate is known to be effective against agitation-induced aggregation of proteins, and a review of the literature regarding the use of polysorbate 80 in chronic intravenous therapies found the proposed level to be included in Aldurazyme™ (10  $\mu$ M/mL) to be well below that used in other pharmaceutical formulations (Bam, *et al.*, *J. Pharm. Sci.* 87(12):1554-9 (1998); Kreilgaard, *et al.*, *J. Pharm. Sci.* 87(12):1597-603 (1998)).

The safety and efficacy of the commercial formulation were assessed in Good Laboratory Practice (GLP) toxicology studies as well as Phase III study.

The invention having been described, the following examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

#### EXAMPLE 1

##### 25 Producing Recombinant $\alpha$ -L-Iduronidase

Standard techniques such as those described by Sambrook, *et al* (*Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1987)) may be used to clone cDNA encoding human  $\alpha$ -L-iduronidase. The human  $\alpha$ -L-iduronidase cDNA previously cloned was subcloned into PRCCMV (InVitrogen) as a HindIII-XbaI fragment from a bluescript KS subclone. An intron cassette derived from the murine immunoglobulin Cot intron between exons 2 and 3 was constructed using PCR amplification of bases 788-1372 (Tucker, *et al.*, *Proc. Natl. Acad.*

*Sci. USA* 78: 7684-7688 (1991) of clone pRIR14.5 (Kakkis, *et al.*, *Nucleic Acids Res.* 16:7796 (1988)). The cassette included 136 bp of the 3' end of exon 2 and 242 bp of the 5' end of exon 3, which would remain in the properly spliced cDNA. No ATG sequences are present in the coding region of the intron cassette. The intron cassette was cloned into the HindIII site 5' of the  $\alpha$ -L-iduronidase cDNA. The neo gene was deleted by digestion with XhoI followed by recircularizing the vector to make pCMVhldu.

One vial of the working cell bank is thawed and placed in three T225 flasks in DME/F12 or PF-CHO plus supplements, plus 5% FBS and 500  $\mu$ g/ml G418. After 2-5 days, the cells are passaged using trypsin-EDTA to a 1-liter spinner flask in the same medium for 2-5 days. The cells are then transferred to two 3-liter spinner flasks for 2-5 days, followed by four 8-liter spinner flasks for 2-5 days. The inoculum from the 8-liter spinner flasks is added to two 110-liter Applikon® stirred tank bioreactors with an 80-90 liter working volume. Macroporous cellulose microcarriers are added at 2 grams per liter (160 grams), with PF-CHO or DME/F12 plus supplements, 5% FBS and 500  $\mu$ g/ml of G418 at a final volume of 80-90 liters. The flask is stirred by an overhead drive with a marine impeller. The culture is monitored for agitation speed, temperature, DO and pH probes and controlled the Applikon® control system with a PC interface. The parameters are controlled at the set points or range, 35-37° C depending on culture conditions, 40% air saturation, and pH 6.95, using a heating blanket, oxygen sparger and base pump. The culture is incubated for 3-5 days at which time the culture is emerging from the log phase growth at  $1-3 \times 10^6$  cells per ml. Thereafter, perfusion is initiated at an increasing rate with PF-CHO medium (with custom modifications, JRH Biosciences). The first four days of collection (range of 3-5 days) are set aside as "washout." The collection thereafter is the beginning of the production run. Production continues with medium changes of as much as 2-3.5 culture volumes per day for 20-36 days. The culture may be extended for 40 days or longer. The culture is monitored for temperature, pH and DO on a continuous basis. The purification of the enzyme proceeds as described above. Collected production medium containing iduronidase is then acidified to pH 5.3, filtered through a 0.2-micron filter and purified using Blue-Sepharose chromatography. The purified enzyme from multiple rounds of Blue-Sepharose chromatography are then pooled and applied to a copper chelating column and eluted with glycerol in the buffer at a pH of 3.7. The enzyme is held at the acidic pH to inactivate potential viruses. The copper column eluate

is then adjusted to pH 5.7 and 2 M NaCl and loaded on the phenyl Sepharose column. The enzyme is eluted at 0.7 M NaCl. The eluate is concentrated and diafiltered into a formulation buffer of 150 mM NaCl, 100 mM NaPO<sub>4</sub>, pH5.8. The enzyme is filtered through a 40 nM filter to remove potential viruses and the filtrate adjusted to 0.001% polysorbate 80. The formulated enzyme is sterilely bulk filled into sterile polyethylene containers. The bulk enzyme is then filtered and filled into 5 cc Type 1 glass vials appropriate for injectable pharmaceuticals, stoppered and capped.

#### EXAMPLE 2

For bioreactors using single cell suspensions, the seed train is prepared as described above in EXAMPLE 1. Using a single cell suspension simplifies bioreactor preparation and inoculation. The bioreactor is inoculated with cells in DMEM/F12 medium (25% of reactor volume) and JRH 325 modified (25% of reactor volume). Medium equal to 50% of the working reactor volume is added over 48 hours. Perfusion (and harvest) is started when cell density reaches  $1.0 \times 10^6$  and the perfusion medium is the same as described above.

#### EXAMPLE 3

Short-term intravenous administration of purified human recombinant  $\alpha$ -L-iduronidase to 9 MPS I dogs and 6 MPS I cats has shown significant uptake of an enzyme in a variety of tissues with an estimated 50% or more recovery in tissues 24 hours after a single dose. Although liver and spleen take up the largest amount of enzymes, and have the best pathologic improvement, improvements in pathology and glycosaminoglycan content has been observed in many, but not all tissues. In particular, the cartilage, brain and heart valve did not have significant improvement. Clinical improvement was observed in a single dog on long-term treatment for 13 months, but other studies have been limited to 6 months or less. All dogs, and most cats, that received recombinant human enzyme developed antibodies to the human product. The IgG antibodies are of the complement activating type (probable canine IgG equivalent). This phenomena is also observed in at least 13% of alglucerase-treated Gaucher patients. Proteinuria has been observed in one dog which may be related to immune complex disease. No other effects of the antibodies have been observed in the other treated animals. Specific toxicity was

not observed and clinical laboratory studies (complete blood counts, electrolytes, BLJN/creatinine, liver enzymes, urinalysis) have been otherwise normal.

Enzyme therapy at even small doses of 25,000 units (0.1 mg/kg/wk) resulted in significant enzyme distribution to some tissues and decreases in GAG storage. If continued for over 1 year, significant clinical effects of the therapy were evident in terms of activity, size and overall appearance of health. The therapy at this dose did not improve other tissues that are important sites for disease in this entity such as cartilage and brain. Higher doses of 125,000 units (0.5 mg/kg) given 5 times over two weeks demonstrate that improved tissue penetration can be achieved and a therapeutic effect at the tissue level was accomplished in as little as 2 weeks. Studies at this increased dose are ongoing in two dogs for six months to date. These MPS I dogs are showing significant clinical improvement and substantial decreases in urinary GAG excretion into the normal range. Other than an immune reaction controlled by altered administration techniques, the enzyme therapy has not shown significant clinical or biochemical toxicity. Enzyme therapy at this higher weekly dose is effective at improving some clinical features of MPS I and decreasing storage without significant toxicity.

The results of these various studies in MPS I dogs and one study in MPS I cats show that human recombinant  $\alpha$ -L-iduronidase is safe. Although these same results provide significant rationale that this recombinant enzyme should be effective in treating  $\alpha$ -L-iduronidase deficiency, they do not predict the clinical benefits or the potential immunological risks of enzyme therapy in humans.

#### EXAMPLE 4

The human cDNA of  $\alpha$ -L-iduronidase predicts a protein of 653 amino acids and an expected molecular weight of 70,000 daltons after signal peptide cleavage. Amino acid sequencing reveals alanine 26 at the N-terminus giving an expected protein of 629 amino acids. Human recombinant  $\alpha$ -L-iduronidase has a Histidine at position 8 of the mature protein. The predicted protein sequence comprises six potential N-linked oligosaccharide modification sites. All of these sites are modified in the recombinant protein. The third and sixth sites have been demonstrated to contain one or more mannose 6-phosphate residues responsible for high affinity uptake into cells.

This peptide corresponds to Amino Acids 26-45 of Human Recombinant  $\alpha$ -L-iduronidase with an N-terminus alanine and the following sequence:

ala-glu-ala-pro-his-leu-val-his-val-asp-ala-ala-arg-ala-leu-trp-pro-leu-arg-arg  
(part of SEQ ID NO:2)

5       The recombinant enzyme has an apparent molecular weight of 82,000 daltons on SDS-PAGE due to carbohydrate modifications. Purified human recombinant  $\alpha$ -L-iduronidase has been sequenced by the UCLA Protein Sequencing facility. It is preferred to administer the recombinant enzyme intravenously. Human recombinant  $\alpha$ -L-iduronidase was supplied for the clinical trial in 10 mL polypropylene vials at a  
10       concentration of 100,000-200,000 units per mL. The final dosage form of the enzyme used in the clinical trial includes human recombinant  $\alpha$ -L-iduronidase, normal saline, and 100 mM phosphate buffer at pH 5.8. These are prepared in a bag of normal saline. Polysorbate 80 at a final concentration of 0.001 % was added to the formulation to stabilize the protein against shear, thereby avoiding precipitation in the final product vials.

15       **Final Vial Formulation Currently in Use**

<u>Component</u>	<u>Composition</u>
$\alpha$ -L-iduronidase	Target to 0.7 mg/mL or 100 (new) units per mL
Sodium chloride solution	150 mM
Sodium phosphate buffer	100 mM, pH 5.8
20   Polysorbate 80	0.001%

**Final Dosage Form Used in the Treatment of Patients**

<u>Component</u>	<u>Composition</u>
$\alpha$ -L-iduronidase product	5-12 fold dilution of vial concentration
25   Sodium chloride solution	50 mM Sodium phosphate buffer 100-250 cc IV bag
Human albumin	1 mg/ml

**EXAMPLE 5**

30       **Phase I Study - Effects of Intravenous Administration of  $\alpha$ -L-Iduronidase in Patients with Mucopolysaccharidosis I (52 weeks)**

Based on studies of cloning of cDNA encoding  $\alpha$ -L-iduronidase (Scott, *et al.*, *Proc. Natl. Acad. Sci. USA* 88: 9695-99 (1991); Stoltzfus, *et al.*, *J. Biol. Chem.* 267:



6570-75 (1992)) and animal studies showing effects of  $\alpha$ -L-iduronidase to reduce lysosomal storage in many tissues (Shull, *et al.*, *Proc. Natl. Acad. Sci. USA* 91: 12937-41 (1994); Kakkis, *et al.*, *Biochem. Mol. Med.* 58: 156-67 (1996)), a 52-week study was conducted to assess the safety and clinical efficacy of intravenous administration of highly purified  $\alpha$ -L-iduronidase in ten patients with mucopolysaccharidosis I (MPS I).

Recombinant human  $\alpha$ -L-iduronidase was produced and purified to greater than 97-99%. Patients demonstrated typical clinical manifestations of the disorder and diagnosis was confirmed by biochemical determination of  $\alpha$ -L-iduronidase deficiency in leukocytes.

Patients were given recombinant human  $\alpha$ -L-iduronidase (diluted in normal saline with 0.1% human serum albumin) intravenously at a dose of 125,000 units per kg (using original assay and unit definition); 3,000 units per kg were given over the first hour, and 61,000 units per kg in each of the following two hours. The dose of 125,000 units per kg is equivalent to 100 SI units per kg using the new assay. The infusions were prolonged up to 4-6 hours in patients who had hypersensitivity reactions.

At baseline and at 6, 12, 26 and 52 weeks depending on the evaluation, the patients underwent examinations including history, physical examinations by specialists, echocardiography, EKG, MRI, polysomnography (weeks 0 and 26), skeletal survey (weeks 0, 26, 52), range of motion measurements, corneal photographs, and skin biopsy (week 0) to set up fibroblast cultures for enzyme determination and genotyping. Range of motion measurements were performed with a goniometer and the maximum active (patient initiated) range was recorded for each motion. Shoulder flexion is movement of the elbow anteriorly from the side of the body and elbow and knee extension represent straightening of the joint. Degrees of restriction represent the difference between the normal maximum range of motion for age and the measured value. Polysomnography was performed according to American Thoracic Society guidelines and apneic events (cessation of oro-nasal airflow for 10 seconds or more), hypopneic events (decreased oro-nasal airflow of 50% or more with desaturation of 2% or more, or evidence of arousal), minutes below 89% oxygen saturation and total sleep time recorded among the standard measurements required. From these data an apnea/hypopnea index was calculated by dividing the total number of apneic and hypopneic events by the number of hours of sleep. Biochemical studies included measurement of enzyme activity in leukocytes and

brushings of buccal mucosal, urinary glycosaminoglycan levels, and tests for serum antibodies to recombinant human  $\alpha$ -L-iduronidase (ELISA and Western blot). Organ volumes were determined by analysis of MRI digital image data using Advantage Windows workstation software from General Electric. The organ volume was measured in milliliters and was converted to weight assuming a density of 1 gram per ml. Urinary glycosaminoglycan excretion was assayed by an adaptation of a published method. Western blots and ELISA assays for antibodies to recombinant human  $\alpha$ -L-iduronidase were performed by standard methods. Uronic acids and N-sulfate of urinary glycosaminoglycans were analyzed by the orcinol, carbazole and MBTH methods, and by electrophoretic separations.

All patients received weekly infusions of recombinant human  $\alpha$ -L-iduronidase administered for 52 weeks. The mean activity of  $\alpha$ -L-iduronidase in leukocytes was 0.04 units per mg before treatment and when measured on average 7 days after an infusion (i.e. immediately before the next infusion), 4.98 units per mg, or 15.0 percent of normal. Enzyme activity was not detectable in buccal brushings prior to treatment, but 7 days after infusions it reached a level of 1 percent of normal.

Liver volume decreased by 19 to 37 percent from baseline in 9 patients and 5 percent in one patient at 52 weeks; the mean decrease was 25.0 percent (n=10, P<0.001). By 26 weeks, liver size was normal for body weight and age in 8 patients (Figure 1). In 20 patients (patients 6 and 9) with the largest relative liver size at baseline, liver size was close to normal at 52 weeks (3.2 and 3.3 percent of body weight, respectively). Spleen size decreased in 8 patients by 13 to 42 percent from baseline (mean decrease of 20 percent in 10 patients, P<0.001).

Urinary glycosaminoglycan excretion declined rapidly by 3 to 4 weeks and by 8-12 weeks had fallen by 60-80 percent of baseline. At 52 weeks, the mean reduction was 63 percent (range 53-74; p<0.001). Eight of ten patients had a 75 percent or greater reduction of the baseline amount of urinary glycosaminoglycan in excess of the upper limit of normal for age. The results were confirmed by assay of uronic acids and N-sulfate (a test specific for heparan sulfate). Electrophoresis studies of urine detected a significant reduction in heparan sulfate and dermatan sulfate excretion but some excess dermatan sulfate excretion persisted in all patients.

The mean height increased 6.0 cm (5.2 percent) in the 6 prepubertal patients (Table 2) and their mean height growth velocity increased from 2.8 cm/yr to 5.2 cm/yr during treatment ( $P=0.011$ ). For all 10 patients, mean body weight increased 3.2 kg (8.8 percent) and the mean increase was 4.2 kg (17.1 percent) for the 6 prepubertal patients (Table 2). In these 6 patients, the mean pretreatment weight growth velocity increased from 1.7 kg per year to 3.8 kg per year during treatment ( $P=0.04$ ).

Shoulder flexion (moving the elbow anteriorly) increased in 6 of the 8 subjects evaluated at baseline with a mean improvement for the right and left shoulders of  $28^{\circ}$  and  $26^{\circ}$ , respectively ( $P < 0.002$ ; Figure 2). Elbow extension and knee extension increased by a mean of  $7.0^{\circ}$  ( $P < 0.03$ ) and  $3.2^{\circ}$  ( $P=0.10$ ), respectively, in the 10 patients (Figure 2).

Analysis of the improvement in individual patients revealed that the most restricted joints had the greatest improvement. For example at baseline, patients 5, 9 and 10 could not flex their shoulders (move the elbow anteriorly) beyond  $100^{\circ}$ , which increased  $21^{\circ}$  to  $51^{\circ}$  after treatment. Similarly, patients 2 and 9 had a substantial increase in knee extension. The changes in range of motion were accompanied by patient-reported increases in physical activities such as being able to wash their hair, hold a hamburger normally, hang from monkey bars, and play sports better.

Seven patients had a decrease in apnea and hypopnea events from 155 to 60 per night upon treatment (a 61 percent decrease) with a change in mean apnea/hypopnea index (total number of events per hour) from 2.1 to 1.0. Three patients had clinically significant sleep apnea and all three improved during treatment. In patient 2, the apnea/hypopnea index decreased from 4.5 at baseline to 0.4 at 26 weeks and total time of oxygen desaturation decreased from 48 minutes to 1 minute per night. Patient 6 required nightly continuous positive airway pressure therapy before treatment due to severe desaturation (61 minutes below 89 percent saturation with continuous positive airway pressure in 368 minutes of sleep), but by 52 weeks, the patient tolerated the sleep study without CPAP and desaturated below 89 percent for only 8 minutes during 332 minutes of sleep. Patient 9 had an apnea hypopnea index of 9.5 which decreased to 4.0 by 26 weeks. Patient 8 worsened with an apnea hypopnea index of 0.1 increasing to 3.1 at 26 weeks and 9.3 at 52 weeks for unclear reasons. Eight of ten patients or their families reported improved breathing, and 5 of 7 noted quieter nighttime breathing, improved sleep quality and decreased daytime somnolence.

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New York Heart Association functional classification was determined by serial patient interviews. All 10 patients reported improvement by one or two classes but there was no significant objective data from echocardiographic studies to verify direct cardiac benefit. The improved functional scores may reflect improvements in other aspects of MPS I disease rather than cardiac function. Comparing baseline to 52 weeks of treatment, echocardiography demonstrated decreased tricuspid regurgitation or pulmonic regurgitation in 4 patients but two patients (patients 2 and 7) had worsening mitral regurgitation. At baseline, patient 6 had atrial flutter and clinical signs of cardiac failure including dyspnea at rest and peripheral edema. By 12 weeks, he had normal sinus rhythm with first degree block and his dyspnea at rest and pitting edema resolved.

All 10 patients reported a lack of endurance and limitations of daily activities before treatment but exercise tolerance was not formally tested. During treatment, all patients improved and by 26 weeks, many were able to walk more, run and play sports. Patients 3, 4 and 5 reported the resolution of severe incapacitating headaches after treatment for 6-12 weeks.

Several patients reported decreased photophobia or conjunctival irritation. Visual acuity improved in one patient (20/1000 to 20/200 in one eye) and modestly in 2 others.

The results of this study indicate that intravenous administration of the highly purified recombinant human  $\alpha$ -L-iduronidase of the present invention results in clinical and biochemical improvement in patients with Mucopolysaccharidosis I. The normalization of liver size and near normalization of urinary glycosaminoglycan excretion is consistent with data from studies in dogs with Mucopolysaccharidosis I, which demonstrated clearance of storage in the liver and decreased urinary glycosaminoglycan excretion in as little as 2 weeks.

Hypersensitivity reactions to the infusions of recombinant human  $\alpha$ -L-iduronidase were less severe than predicted from studies in dogs. Though important in some patients, recurrent urticaria was manageable with premedication and adjustments in infusion rate. Antibodies specific to  $\alpha$ -L-iduronidase were detected in 4 patients with usually subclinical complement activation, and both the antibodies and complement activation declined with time. Similar IgG-mediated immune responses have been previously noted in patients with Gaucher disease treat with glucocerebrosidase, although the events were

more frequent in our patients. Mucopolysaccharidosis I patients with a null genotype may have a greater immune response than in these 10 patients, none of whom has a null.

Thus, recombinant human  $\alpha$ -L-iduronidase can reduce lysosomal storage and ameliorates some aspects of clinical disease of Mucopolysaccharidosis I.

5

#### EXAMPLE 6

##### Phase III Study - Effects of Intravenous Administration of $\alpha$ -L-Iduronidase in Patients with Mucopolysaccharidosis I (26 weeks)

A multi-national, multi-center, double-blind, randomized, placebo-controlled  
10 study was conducted to further assess the safety and clinical efficacy of intravenous administration of highly purified  $\alpha$ -L-Iduronidase in 45 MPS I patients.

Recombinant human  $\alpha$ -L-Iduronidase was purified to greater than 99%. The patients were characterized by age of at least five years old, less than 10 percent of normal enzyme activity, a baseline forced vital capacity (FVC) reflecting pulmonary function of  
15 80% or less of percent predicted normal, and a capability of standing for 6 minutes and walking at least 5 meters. Of the 45 patients, 22 patients were treated with highly purified  $\alpha$ -L-Iduronidase and the remaining 23 received a placebo. Patients were administered human  $\alpha$ -L-Iduronidase intravenously at a dose of 100 units per kilogram via a 4-hour intravenous infusion each week for 26 weeks.

##### 20 *Efficacy Endpoints*

Patients were assessed by measuring primary efficacy endpoints, the change from baseline to week 26 in the % FVC and a six-minute walk distance using the Wilcoxon Rank Sum Test. Patients were further assessed by secondary efficacy endpoints including apnea/hypoxia index (sleep study), liver organ volume (hepatomegaly), disability score  
25 index (Child Health Assessment Questionnaire / Health Assessment Questionnaire, CHAQ/HAQ), and shoulder flexion reflecting joint range of motion. These endpoints were measured as a change in baseline to week 26 by the Analysis of Variance test. Patients were also assessed by measuring tertiary efficacy endpoints, including urinary glucosaminoglycan (GAG) levels, totally respiratory event index (sleep study), pain scale  
30 (CHAQ), shoulder extension, knee extension and flexion, quality of life (50-question Child Health Questionnaire Physical Functioning, CHQ PF 50; 87-question Child Health Questionnaire directed to the child with questions combined to create concepts, CHQ

CF87; 36-question Short Form Health Status Survey, SF-36), growth in prepubertal only, visual acuity, echocardiogram, force expiratory volume (FEV<sub>1</sub>), and investigator global assessment. The investigator global assessment comprises a series of seven categories in which the investigator is providing an assessment as to how each patient is improving during the trial.

#### *Safety Endpoints*

Safety was assessed by measurement of the frequency of adverse events, serious adverse events, and infusion-associated reactions, immunogenicity testing, and measurement of other safety parameters by physical examinations, testing of vital signs, brain/cranio-cervical junction magnetic resonance imaging (MRI), and standard laboratory evaluations.

### Results

#### *Efficacy Endpoints*

With respect to primary efficacy endpoints, a statistically significant difference ( $p = 0.028$ ) was seen in the change in % predicted FVC (see Table I). A close to statistically significant difference ( $p = 0.066$ ) was noted in the change in 6-minute walk (Table II).

Although there was no significant overall difference observed in the sleep apnea/hypopnea index, a reduction of events was observed in enzyme-treated patients with clinically significant disease ( $n = 6/9$ ,  $p = 0.011$ ). Consistent with the prior study, there was a significant reduction in liver volume ( $p = 0.001$ ) and hence improvement in occurrence of hepatomegaly. There were no significant differences in CHAQ/HAQ Disability Index or Joint Range of Motion, although there was a trend towards improvement in more severe patients. There was a statistically significant rapid reduction in urinary GAG ( $p < 0.001$ ). Trends in favor of enzyme treatment were noted in measurements of right shoulder extension, left knee flexion, and LVDS (Left Ventricle Internal Dimension at End-Systole in cm) as measured by echocardiography.

Table I  
Percent Predicted Change From Baseline

Intent To Treat Population				
	Baseline	Week 26	Change	Difference from Placebo

	(% Predicted)	(% Predicted)		
$\alpha$ -L-Iduronidase (Aldurazyme™) n = 22	48.4 $\pm$ 14.85	50.2 $\pm$ 17.10	1.8 $\pm$ 7.70	4.5 p = 0.028
Placebo n = 23	54.2 $\pm$ 16.00	51.5 $\pm$ 13.13	-2.7 $\pm$ 7.12	

Table II  
Six-Minute Walk Change from Baseline

5

Intent To Treat Population

	Baseline (m)	Week 26 (m)	Change	Difference from Placebo
$\alpha$ -L-Iduronidase (Aldurazyme™) n = 22	319.0 $\pm$ 131.41	338.8 $\pm$ 127.06	19.7 $\pm$ 68.56	38.1 p = 0.066
Placebo n = 23	366.7 $\pm$ 113.68	348.3 $\pm$ 128.81	-18.3 $\pm$ 67.49	

*Comparison with Phase I Study*

The results from measurement of secondary and tertiary endpoints were consistent with that of the Phase I study. For example, in both studies there was a significant reduction in liver volume (p = 0.001). Liver volume recovered to normal in almost 60% of enzyme-treated patients in the Phase III study at the end of 26 weeks. Similarly, in the Phase I study, liver size was normal for body weight and age in eight of ten patients by 26 weeks. In both studies, there was a reduction in sleep apnea/hypopnea events. As described above, in the Phase III study, a reduction in events (p=0.011) was observed in six of nine enzyme-treated patients with clinically significant disease. Similarly, seven of ten patients in the Phase I study showed a decrease in apnea and hypopnea events from 155 to 60 per night upon treatment with a reduction in the mean apnea/hypopnea index.

There was also an improvement in the joint range of motion of more severe patients treated with the enzyme. Urinary GAG excretion rapidly declined in enzyme-treated patients in both studies. In the Phase III study, there was a statistically significant rapid reduction in urinary GAG levels ( $p < 0.001$ ). Similarly, in the Phase I study, urinary GAG excretion declined rapidly by three to four weeks and by eight to twelve weeks had fallen by 60 – 80% of baseline. Thus, there appeared to a strong correlations in the secondary and tertiary efficacy endpoints of Phase I and III studies.

The results show that  $\alpha$ -L-Iduronidase appears to be safe and well-tolerated. The types of adverse events were similar between days of infusion and non-infusion days. The frequency of infusion – associated reactions was similar between placebo and enzyme-treated groups. With respect to immunogenicity testing of IgG, all 22 patients in the enzyme-treated group seroconverted with a mean time to seroconversion of 41 days. Seroconversion did not result in increased infusion-associated reactions or other adverse events. Among three patients tested for IgE, including one patient from the placebo group and two enzyme-treated patients, all IgE tests were negative. There were no clinically significant changes in observations from physical examination, vital, and brain / cranio – cervical junction MRI from baseline to week 26. Standard laboratory evaluations showed: (1) no significant laboratory changes indicating a negative treatment effect; (2) a significant increase in platelet counts in enzyme – treated patients; and (3) improvement in liver enzyme abnormalities in enzyme-treated patients.

#### *Summary*

In the Phase I studies i) all patients developed antibodies to the treatment with all 10 to contaminating proteins and 4 to IDU itself; ii) 5 patients (50%) had clinical manifestations of an allergic response of which the most common urticaria (hives); and iii) several of these reactions were classified as serious adverse events (SAEs) (meaning they required medical intervention) related to treatment with  $\alpha$ -L-Iduronidase .

In the Phase III study, i) all patients developed antibodies to the treatment but it is not yet known whether these were to CP or IDU itself; ii) clinical manifestations of an allergic response were mild in all patients and were comparable between the placebo and  $\alpha$ -L-Iduronidase treated groups; iii)



there were no SAEs considered related to treatment with  $\alpha$ -L-Iduronidase; and iv) there was no urticaria reported.

In summary, the efficacy data gathered in the MPS I dog studies and the two human clinical trials tells a consistent story of improvement in disease symptoms. The safety profile of the product improved significantly in the Phase III versus the Phase I. This corroborates the theory that the material of increased purity used in the Phase III trial is an improvement over the material used in the Phase I trial.

The invention, and the manner and process of making and using it, are now described in such full, clear, concise and exact terms as to enable any person skilled in the art to which it pertains, to make and use the same. It is to be understood that the foregoing describes preferred embodiments of the present invention and that modifications may be made therein without departing from the spirit or scope of the present invention as set forth in the claims. To particularly point out and distinctly claim the subject matter regarded as invention, the following claims conclude this specification.